

Control of *Phytophthora cryptogea* in the hydroponic forcing of witloof chicory with the rhamnolipid-based biosurfactant formulation PRO1

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Rhamnolipids, extracellular metabolites of *Pseudomonas aeruginosa* with surfactant properties, proved to be very effective in controlling the spread of brown root rot disease caused by *Phytophthora cryptogea* in the hydroponic forcing system of witloof chicory (*Cichorium intybus* var. *foliosum*). The biosurfactant was applied as the product PRO1, a formulation of 25% rhamnolipids in oil. Both an *in vitro* screening and *in vivo* experiments in a mini-hydroponic system demonstrated the ability of PRO1 to control brown root rot. A 25 µg mL⁻¹ rhamnolipids nutrient solution was enough to obtain good control of an artificial infection with a zoospore suspension of *P. cryptogea*. The biosurfactant PRO1 performed well in a semicommercial system under growers' conditions. A treatment of 25 µg mL⁻¹ rhamnolipids (100 µg mL⁻¹ PRO1) reduced the disease incidence significantly in two independent experiments. However, PRO1 was not effective when a mycelial suspension was used as inoculum. Rhamnolipids have good potential to limit the spread of *P. cryptogea* in the hydroponic forcing system of witloof chicory, and can be used as a preventive measure against brown root rot.

Keywords: biological control, *Cichorium intybus* var. *foliosum*, *Pseudomonas aeruginosa*, soil-free growing system, zoospore pathogens

Introduction

Witloof chicory (*Cichorium intybus* var. *foliosum*) is an important vegetable crop in Belgium, France and the Netherlands. The crop, also called Belgian endive, refers to the blanched, tight heads produced by placing (in darkness) big mature chicory roots in forcing structures. Nowadays, the forcing of the crop usually takes place in a hydroponic system.

Phytophthora cryptogea is a zoospore-producing pathogen that causes an important root rot disease of witloof chicory (Forlot *et al.*, 1966; Stanghellini & Kronland, 1982). The pathogen is normally brought into the recirculating system via field soil adhering to the roots or via roots with latent infections. Once the pathogen is introduced into such a cultural system, its control is often difficult and commonly requires the shutdown and disinfection of the entire system (Stanghellini *et al.*, 1996b). Early detection and immediate proper action are therefore very important. Currently, effective detection

methods for *P. cryptogea* in a hydroponic system are available (Pettitt *et al.*, 2002), but control measures are still traditional using commonly registered pesticides such as fosetyl-aluminium, propamocarb hydrochloride and dimethomorph.

Tomlinson & Faithfull (1979; 1980), Stanghellini & Miller (1997) and Demeulenaere & Höfte (2000) have shown that surfactants can exhibit a lytic activity against zoospores. The effectiveness of surfactants as practical control agents for root-infecting zoospore plant pathogens in hydroponic systems has been demonstrated by Stanghellini & Rasmussen (1994) and Stanghellini *et al.* (1996a,b, 2000). Surfactants are surface-active compounds capable of reducing surface and interfacial tension at the interfaces between liquids, solids and gases, thereby allowing them to mix or disperse readily as emulsions in water or other liquids (Banat *et al.*, 2000). Microorganisms produce a variety of surface-active agents (biosurfactants). Biosurfactants have a hydrophilic portion, consisting of mono-, oligo- or polysaccharides, amino acids, peptides, carboxylate or phosphate groups, and a hydrophobic portion, which is composed of saturated or unsaturated hydroxyl (fatty) acids or fatty alcohols (Lang & Wullbrandt, 1999). These biosurfactants can be further divided into low-molecular-weight molecules that lower

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surface and interfacial tensions efficiently and high-molecular-weight polymers that bind tightly to surfaces (Ron & Rosenberg, 2001). Tensio-active glycolipids containing one (mono-rhamnolipid) or two (di-rhamnolipid) L-rhamnose molecules and β -hydroxydecanoic acid are called rhamnolipids (Rahim *et al.*, 2001).

The use of biosurfactant-producing organisms in the control of plant diseases in general, and zoospore plant pathogens in particular, has not been extensively studied. Nielsen *et al.* (1998, 1999, 2002), Nielsen & Sørensen (2003) and Thrane *et al.* (2000) reported a cyclic lipopeptide, viscosinamide, produced by a *Pseudomonas fluorescens* strain with surfactant and antifungal properties, while de Souza *et al.* (2003) described *Ps. fluorescens* strains that produce surfactants with lytic activity against zoospores of several oomycetes. Stanghellini & Miller (1997) evaluated the potential of various rhamnolipid-producing fluorescent *Pseudomonas* spp. in the control of *P. capsici* on peppers grown in hydroponic units. In a recent study on the biological control of *Pythium aphanidermatum* in soil-free systems, Folman *et al.* (2003) studied the role of biosurfactants in the control of various oomycete pathogens by *Lysobacter enzymogenes*.

In this work, the capacity of a selected biosurfactant formulation PRO1, containing rhamnolipids from *Ps. aeruginosa*, is evaluated to control *Phytophthora* root rot in witloof chicory hydroponic forcing.

Materials and methods

Plant material

Witloof chicory (*C. intybus* var. *foliosum*) cultivars used in this study were Vintor and Platine. There is no difference in disease resistance against *P. cryptogea* brown root rot between these cultivars. Organically grown roots were supplied by the Provincial Research and Advisory Centre for Agriculture and Horticulture, POVLIT Rumbek-Beitem, Belgium, and stored at 0–1°C until use.

Pathogen

Phytophthora cryptogea strain no. PD2009, isolated from witloof chicory, was supplied by 'Plantenziektenkundige Dienst', Wageningen, the Netherlands. Axenic cultures of the strain were grown in darkness at 24°C on V8 juice agar [V8A; 200 mL V8-juice (Campbell Foods Belgium, Puurs, Belgium), 15 g agar (Pulvis; Federa, 1130 Brussels, Belgium), 2 g CaCO₃ and 800 mL distilled water].

Zoospore production

Zoospores were produced under aseptic conditions using a modification of the procedure described by Chen & Zentmyer (1970). Ten 5-mm agar plugs were transferred from the leading edge of a 4-day-old colony to a 90-mm-diameter Petri dish containing 15 mL sterile mineral salt solution (0.145 g Ca(NO₃)₂·4H₂O, 0.012 g MgSO₄·7H₂O, 0.099 g KH₂PO₄ and 0.016 g FeCl₃·6H₂O in 1 L of

distilled water autoclaved for 15 min at 121°C). The Petri dishes were then kept under continuous light at 22°C to induce sporangia formation. After 72 h, the plates were kept at 4°C for 1 h followed by 1 h incubation at room temperature to induce a synchronized zoospore release. The zoospore suspension from each dish was harvested. The plates were rinsed with cold (4°C) sterile distilled water. This water was collected in the same tubes containing the zoospore suspension from the corresponding Petri dish. The inoculum concentration was adjusted by diluting the obtained zoospore suspension to a final concentration of 10² or 10³ zoospores per mL nutrient solution.

Surfactant

The biosurfactant PRO1 consists of a formulation of 25% rhamnolipids (extracted from the bacterium *Ps. aeruginosa*) in oil, and was supplied by Plantsupport (the Netherlands). Concentrations of 12.5 and 25 $\mu\text{g mL}^{-1}$ rhamnolipids (50 and 100 $\mu\text{g mL}^{-1}$ PRO1, respectively) were used in the experiments.

In a pot assay, the surfactant was only added at the beginning of the experiment, whereas for experiments in a hydroponic system, the surfactant applications were given at the beginning of the experiment and repeated after 1 week. Preliminary studies showed that PRO1 at 100 $\mu\text{g mL}^{-1}$ was not phytotoxic (data not shown).

In vivo assays for surfactant testing

Pot assays

Organically grown witloof chicory roots (cv. Vintor) were harvested, stored and prepared for the forcing phase in standardized optimal conditions. The roots were individually placed in pots (10 × 10 × 12 cm) and each pot was filled with 500 mL nutrient solution consisting of 0.46 g L⁻¹ KNO₃, 0.35 g L⁻¹ MgSO₄·7H₂O, 1.00 g L⁻¹ Ca(NO₃)₂·4H₂O, 0.21 g L⁻¹ KH₂PO₄ and 0.30 g L⁻¹ K₂SO₄ diluted in distilled water. In the pot experiments, an inoculum of two zoospore concentrations (10² and 10³ mL⁻¹) of *P. cryptogea* and a control were combined with three surfactant treatments: 0, 50 and 100 $\mu\text{g mL}^{-1}$ PRO1. In each pot experiment, eight replications per treatment were arranged in a complete randomized block design (8 × 3 × 3). In each experiment, infected and healthy control treatments were included. All experiments were conducted in the dark at an air temperature of 18 ± 2°C. Disease incidence (% diseased roots) and disease severity (mean lesion length in cm) were recorded 14 days after inoculation.

Mini-hydroponic system

Organically grown witloof chicory roots (cv. Vintor) were harvested, stored and prepared for the forcing phase in standardized optimal conditions. Twenty-four witloof chicory roots were equally divided over three levels in a mini-hydroponic system (Fig. 1). The container size in the mini-hydroponic system was 40 × 60 cm and had an overflow system at a height of 5 cm. The mini-hydroponic system contained 60 L recirculating nutrient solution (cfr.



Figure 1 Mini-hydroponic system for forcing witloof chicory roots.

pot assays). For the infection, an inoculum density of 10^2 zoospores of *P. cryptogea* was used. PRO1 was applied in concentrations of 50 and $100 \mu\text{g mL}^{-1}$. In each experiment, uninoculated and inoculated control treatments were included. All experiments were conducted in the same conditions as the pot experiments, and disease incidence (% diseased roots) and disease severity (mean lesion length in cm) were recorded 14 days after the start of the experiment. Water flow rate in the mini-hydroponic system was 200 L h^{-1} . The experiments in the mini-hydroponic setup were carried out with an application of the surfactant at the start of the experiment and a repetition after 7 days. Infection with the pathogen was done with a single addition of the inoculum at the start of the experiment. The experiment was repeated.

Hydroponic forcing system 'bain-marie' type (Fig. 2)

Two standardized experiments in a semicommercial system under good experimental practice (GEP) regulations were carried out at the Provincial Research and Advisory Centre for Agriculture and Horticulture in Rumbek-Beitem, Belgium. Organically grown witloof chicory roots were harvested, stored and prepared for the forcing phase in standardized optimal conditions.

In the first experiment, treatments included an uninoculated control treatment, an inoculated treatment and an inoculated treatment in which the nutrient solution was amended with $100 \mu\text{g mL}^{-1}$ PRO1. Water and air temperature were set at 15°C . Roots of cv. Platine were used. The storage period was 4 months.

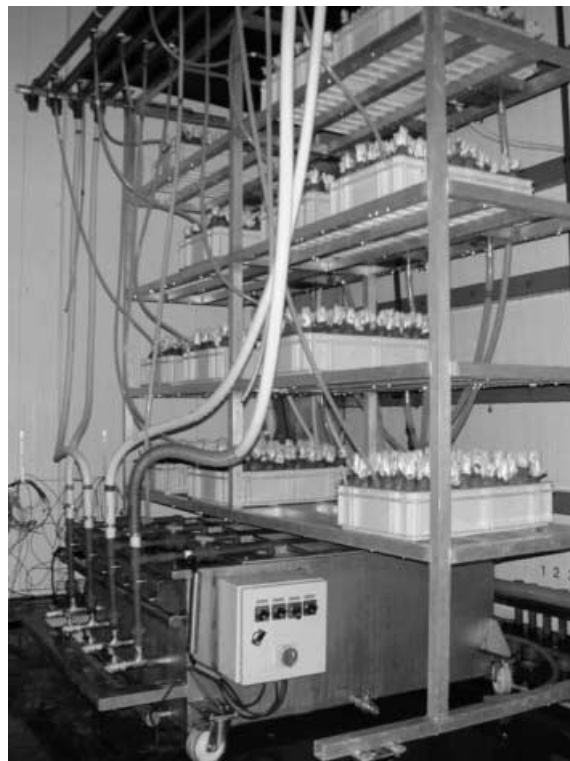


Figure 2 Hydroponic system – 'bain-marie' type for forcing witloof chicory roots.

A second experiment included an uninoculated control treatment, an inoculated control treatment, an inoculated treatment amended with $100 \mu\text{g mL}^{-1}$ PRO1 and an inoculated treatment with an extra 10 diseased roots per tray, amended with $100 \mu\text{g mL}^{-1}$ PRO1. Water and air temperature were set at 17°C . Roots of cv. Vintor were used. The storage period was 4 months.

Infection was carried out with 10^2 zoospores of *P. cryptogea* per mL nutrient solution. Each treatment was carried out in four levels of $40 \times 60 \text{ cm}$ containers containing ~100 roots. The closed recirculating system contained 60 L of nutrient solution (content in mmol L^{-1} : K^+ , 8.0; Mg^{2+} , 1.4; Ca^{2+} , 4.2; NO_3^- , 13.7; PO_4^- , 1.5 and SO_4^- , 2.0). Electric conductivity during the experiment was held constant at $2 \pm 0.2 \text{ mS cm}^{-1}$ and pH was set between 6.5 and 7.5. The amount of nutrient solution in each container was constant by means of an overflow (5 cm high) to the underlying level. The reservoirs of each closed system (one for each treatment) were placed in the same water tank (total volume 1000 L) buffering the water temperature in all treatments (bain-marie system, Fig. 2). Water flow rate was set at 500 L h^{-1} . Nineteen days after placing the roots in the forcing trays, disease incidence (%), yield (kg) and standard quality characteristics [size, compactness, shape, colour and physiological disorders (internal browning)] of the harvested crop were recorded. Biological efficiency (%), which is output (yield in kg) divided by input (average root weight in kg) was calculated.

The date of harvesting was determined by crop maturity and commercial considerations.

Statistical data analysis

Statistical analysis of data was performed using the software package SPSS 11.0.1. (SPSSinc, Chicago, IL, USA). For the nominal data of disease severity (lesion length in cm) the conditions of normality and homogeneity of variances were not fulfilled and a suitable transformation was not available. For these data sets, the nonparametric Kruskal–Wallis and Mann–Whitney tests were performed and α -values were corrected for multiple comparisons. For the ordinal disease incidence data, a logistic regression analysis was performed. Interaction between the different factors was checked.

Results

In vitro experiments

The biosurfactant concentrations used in this study were selected after an initial *in vitro* screening experiment at a wide concentration range. A significant reduction of sporangia formation and general zoospore lysis (within 1 min after surfactant application) was obtained at a minimal concentration of 50 $\mu\text{g mL}^{-1}$ PRO1. Amendment of a solid medium with different concentrations (0–5 mg mL^{-1}) of PRO1 had limited effects on the mycelial growth of *P. cryptogea* (data not shown). Preliminary studies also showed that PRO1 at 100 $\mu\text{g mL}^{-1}$ was not phytotoxic (data not shown).

Pot assays

The infected control treatments only showed a concentration effect for disease severity, and not for disease incidence. An infection with 10^3 zoospores mL^{-1} resulted in 87.5% diseased roots and a mean lesion length of 7.0 cm, compared with an identical disease incidence and a mean lesion length of 3.8 cm at the lower infection pressure of 10^2 zoospores. In the uninfected control treatment, 12.5% of the roots showed phytophthora root rot symptoms with a mean lesion length of 0.4 cm in the roots with brown root rot symptoms (Fig. 3a and b). Adding 50 $\mu\text{g mL}^{-1}$ PRO1 to the pots at a disease pressure of 10^2 zoospores mL^{-1} also resulted in 12.5% diseased roots and a mean lesion length of 5.2 cm. Increasing the surfactant concentration to 100 $\mu\text{g mL}^{-1}$ at the same disease pressure resulted in 25% of the roots with brown root rot symptoms and a mean lesion length in the diseased roots of only 0.7 cm (Fig. 3a and b).

At a higher disease pressure of 10^3 zoospores mL^{-1} , 75 and 37.5% diseased roots were recorded for treatments with 50 and 100 $\mu\text{g mL}^{-1}$ PRO1, respectively (Fig. 3a). The mean lesion length in the roots with disease symptoms for both treatments was significantly reduced to 2.4 and 1.7 cm, respectively (Fig. 3b).

Experiments were repeated with similar results, except for disease severity at the 10^2 zoospores mL^{-1} level (Fig. 3d). For this treatment, no diseased roots at all were recorded in repetition 2 (Fig. 3c and d), while in repetition

1 the few diseased roots were heavily infested, resulting in relatively high mean lesion length (Fig. 3a and b).

In another series of pot experiments, different concentrations of mycelium suspension of *P. cryptogea* were used as inoculum. Only a limited effect on both disease incidence and disease severity was seen after application of PRO1 at a concentration of 50 or 100 $\mu\text{g mL}^{-1}$ (data not shown).

Mini-hydroculture assay

Adding 50 or 100 $\mu\text{g mL}^{-1}$ PRO1 to a mini-hydroponic system infected with 10^2 *P. cryptogea* zoospores mL^{-1} , reduced the disease incidence significantly from 91.6% to 50.0 or 29.2%, respectively (Fig. 4a, repetition 1). A mean lesion length of 3.8 cm in the diseased roots of the infected control was recorded (Fig. 4b, repetition 1). Amendment with 50 or 100 $\mu\text{g mL}^{-1}$ PRO1 reduced the mean lesion length in the diseased roots significantly to an average of 1.0 or 0.8 cm, respectively. This was not significantly different from the healthy control (0.8 cm).

The experiment was repeated with similar results, except for a much higher mean lesion length for the 100 $\mu\text{g mL}^{-1}$ PRO1 treatment. The disease incidence in repetition 2 was very low for this treatment, but the few diseased roots were severely affected, resulting in a high disease severity (Fig. 4a and b, repetition 2).

An experiment with a single application of the surfactant at start-up did not reduce the disease incidence and severity significantly (data not shown).

Hydroponic forcing system ‘bain-marie’ type

In a first experiment in semicommercial conditions, the disease incidence in the inoculated control was only 16.3%. The inoculated treatment with two weekly applications of 100 $\mu\text{g mL}^{-1}$ PRO1 resulted in only three diseased roots out of a total of 400 (0.8%). This was not significantly different from the uninoculated control containing one diseased root (0.3%) (Table 1, experiment 1).

The experiment was repeated with a higher air and water temperature and disease incidence increased to 79.1% in the inoculated treatment. Only 5.5% of the roots showed symptoms of a *P. cryptogea* infection in a treatment with zoospore infection and 100 $\mu\text{g mL}^{-1}$ PRO1. In the uninfected control, 1.7% of the roots showed disease symptoms. In the infected control treatment, a significant reduction in yield (kg) and biological efficiency (%) was observed. The PRO1 treatment did not result in significant yield differences with the uninfected control treatment (Table 1). No significant differences between the different tray levels were observed (data not shown). In the same experiment, a treatment with zoospore infection, 100 $\mu\text{g mL}^{-1}$ PRO1 and an extra 10 diseased roots per tray resulted in 46.4% of the roots with disease symptoms. This is significantly higher than a PRO1 treatment with only a zoospore infection in the nutrient solution. In this treatment, it was observed that mainly roots in direct contact with the diseased roots developed disease symptoms. Additionally, disease can also spread by contact between

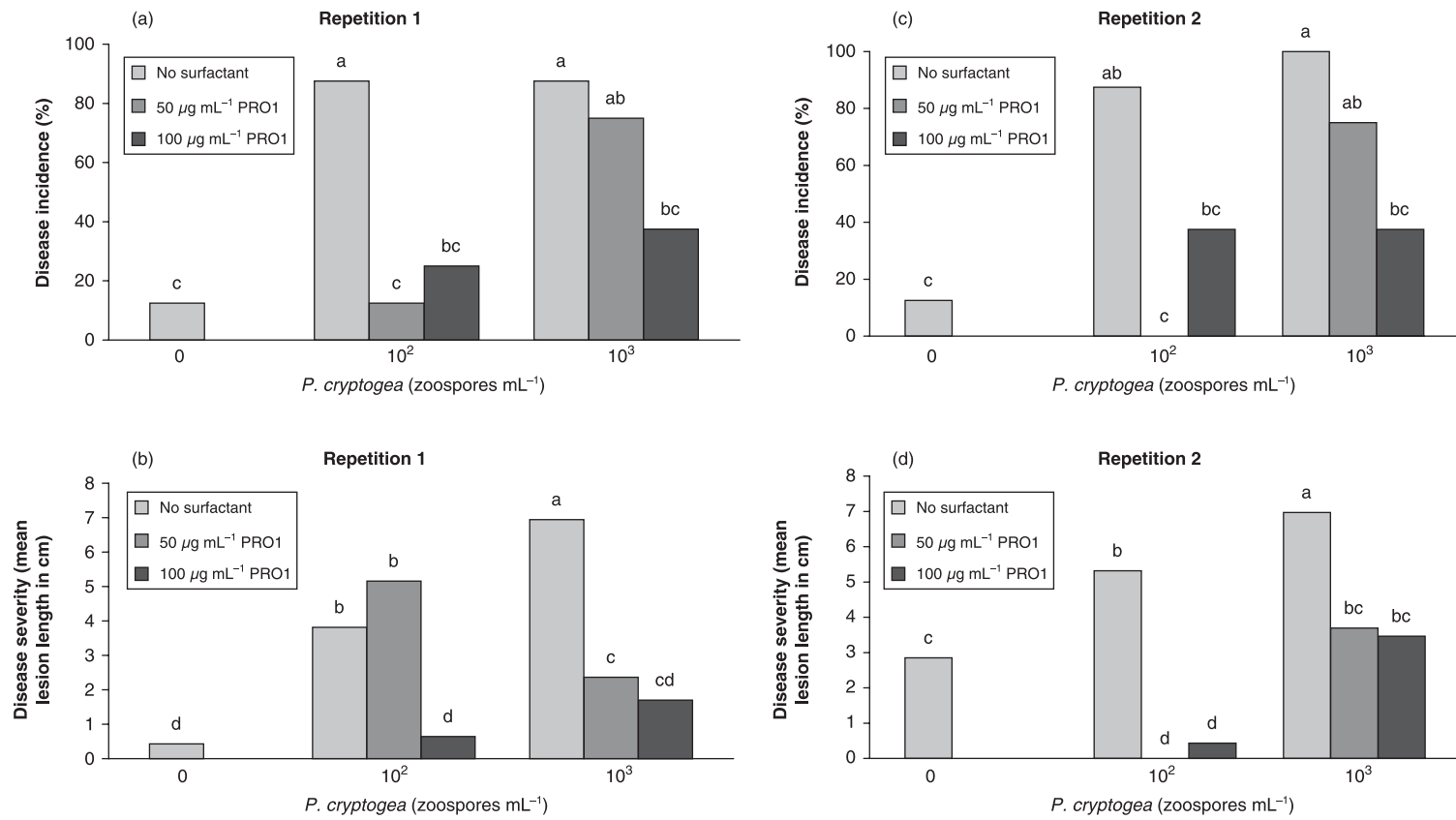


Figure 3 Disease incidence (%) (a, c) and disease severity (mean lesion length of the diseased roots in cm) (b, d) in pot assays for treatments with 50 and 100 µg mL⁻¹ PRO1 and two different infection levels of *Phytophthora cryptogea* (1×10^2 and 1×10^3 zoospores mL⁻¹).

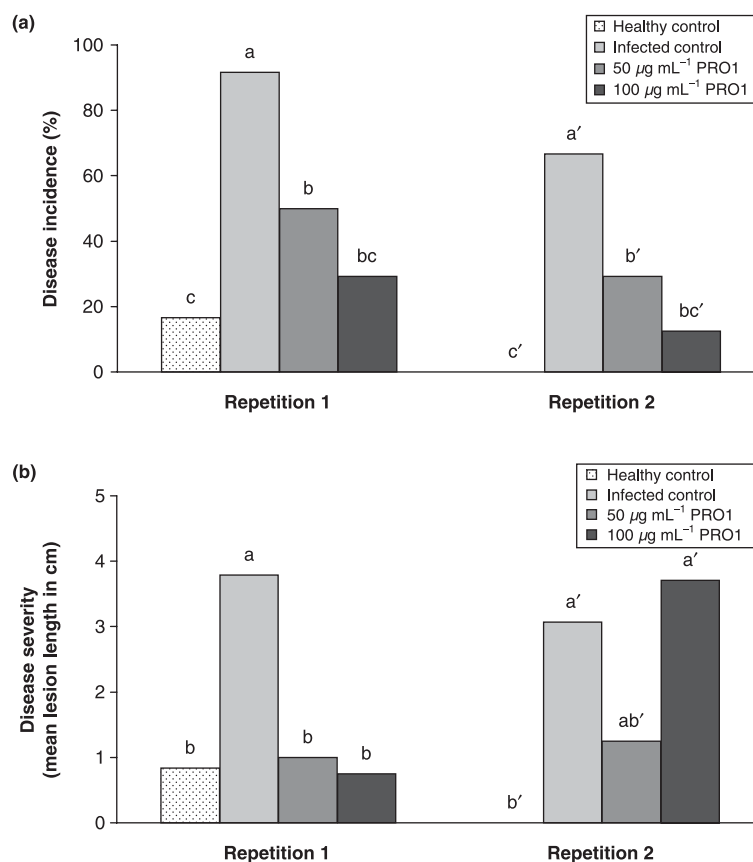


Figure 4 Disease incidence (%) (a) and disease severity (mean lesion length of the diseased roots in cm) (b) in a mini-hydroponic system for treatments with 50 and 100 µg mL⁻¹ PRO1 and infection level of 1×10^2 zoospores *Phytophthora cryptogea* per mL.

Table 1 Disease incidence (%), yield (kg), average root weight (kg) and biological efficiency (%) in a hydroponic system under semicommercial conditions following infection with 1×10^2 zoospores mL⁻¹ of *Phytophthora cryptogea*. PRO1 (100 µg mL⁻¹) was applied at the start of the experiment and the application was repeated after 7 days. For each experiment, values followed by the same letter are not significantly different at $P = 0.05$

	Treatment	Total no. of roots	Yield (kg)	Root weight (kg)	Biological efficiency (%)	Disease incidence (%)
Experiment 1 ^a	Healthy control	400	46.6 a	74.5 a	62.5 a	0.3 b
	Infected control	400	46.2 a	77.1 b	60.0 b	16.3 a
	Infection + PRO1	400	47.6 a	75.0 a	63.5 a	0.8 b
Experiment 2	Healthy control	419	48.5 a	74.2 a	65.4 a	1.7 B
	Infected control	406	44.5 B	72.9 A	61.1 B	79.1 A
	Infection + PRO1	402	48.0 A	74.4 A	64.5 A	5.5 B

^aWater and air temperature was 15°C in experiment 1, and 17°C in experiment 2.

diseased and healthy roots above the water level, where there is no contact with the applied rhamnolipids. Unfortunately, because of technical limitations (the system can only carry a maximum of four treatments), an inoculated control treatment including diseased roots at the start of the experiment could not be included.

Discussion

This study shows that rhamnolipids have the potential to be used in the control of brown root rot caused by *P. cryptogea* in the hydroponic forcing of witloof chicory.

The *in vitro* screening results confirm the results from Stanghellini & Miller (1997), who found zoosporicidal activity of purified mono- and di-rhamnolipids against *P. capsici*, *Pythium aphanidermatum* and *Plasmopara lactucae-radici* at concentrations ranging from 5 to 30 µg mL⁻¹. A range of synthetic surfactants were screened by Demeulenaere & Höfte (2000) on their *in vitro* effects on mycelium growth, sporangia formation and zoospore survival. Their results show significant effects of some surfactants on mycelium growth of *P. cryptogea* from 100 µg mL⁻¹ onwards, and effects on sporangia formation and zoospore production for most surfactants included in the experiments at a concentration as low as 10 or 20 µg mL⁻¹.

In vivo, in all types of experiments, the use of untreated, organically grown witloof chicory roots led to a certain disease level in the uninfected control. This is most likely caused by a natural pathogen infection brought in with the roots from the field.

A good control of the pathogen was obtained with $25 \mu\text{g mL}^{-1}$ rhamnolipids nutrient solution. This also corresponds with the results of Stanghellini & Miller (1997) who used rhamnolipid-producing *Pseudomonas* strains to control *P. capsici* root rot in a hydroponic system. They observed a significant reduction of the disease symptoms at an estimated quantity of $8\text{--}10 \mu\text{g mL}^{-1}$ rhamnolipids. The growth and maintenance of a critical bacteria concentration in such a system, however, require the addition of a C-source to the nutrient solution. Moreover, the regulation of rhamnolipid production in *Pseudomonas* spp. is subject to quorum sensing (Rahim *et al.*, 2001) and is partially dependent on RpoS, the sigma factor of the stationary phase (Medina *et al.*, 2003). These problems can be avoided by using pure rhamnolipids rather than rhamnolipid-producing bacteria. Kim *et al.* (2000) also used pure rhamnolipids to control *Phytophthora* blight in pepper-growing fields. However, leaves and stems of pepper had to be sprayed with a concentration of $500 \mu\text{g mL}^{-1}$ rhamnolipids to obtain a similar control level as obtained with conventional fungicides.

In the present work, one application of rhamnolipids was not sufficient. This corresponds with studies of Stanghellini *et al.* (1996a,b) where fast surfactant degradation was observed and re-application was indispensable. Since a forcing cycle of witloof chicory only takes about 2–3 weeks, the biosurfactant treatments can be limited to two applications.

The low disease level (16.3%) in the infected control of the first semicommercial GEP experiment could be due to the low water and air temperatures (both held constant at 15°C). In the second semicommercial experiment, in which the water and air temperatures were increased to 17°C , the disease incidence in the infected control was 79.1%. A temperature of $18 \pm 2^{\circ}\text{C}$ always resulted in a high disease incidence in the mini-hydroponic system.

The two experiments in the semicommercial system were carried out with witloof chicory roots that had been stored at $0\text{--}1^{\circ}\text{C}$ for 4 months. This may also have played a role, since no problems with artificial infection for similar experiments with synthetic surfactants were seen at lower forcing temperatures (15°C) when roots were used which came straight from the field or underwent only a short storage period (KDJ *et al.*, unpublished data).

Rhamnolipids only had a limited effect on mycelium development (*in vitro* screening results, data not shown). This was confirmed through *in vivo* experiments. PRO1 was not able to control brown root rot disease following an artificial infection with mycelial suspensions of *P. cryptogea*. Additionally, a treatment including ~10% diseased roots in the system showed that a large part of the efficiency of the biosurfactant was lost. Diseased roots could still spread the disease to healthy roots in direct contact, most likely through mycelium, also because part of the

contact zone between diseased and healthy tissue is situated above the water level.

Rhamnolipids thus mainly act against the zoospore stage of the pathogen. It has been observed, however, that the synthetic surfactant Atplus MBA1301, an alcohol alkoxyate, is able to control infections with both mycelium and zoospore suspensions of *P. cryptogea* (KDJ *et al.*, unpublished data).

In conclusion, application of $25 \mu\text{g mL}^{-1}$ rhamnolipids nutrient solution controls the spread of zoospores in the system (preventive control), but once the pathogen is introduced in the root, only a limited effect on the outgrowth of the pathogen is obtained (curative control). In practice, however, zoospores are the most important infection structures in hydroponic growing systems. Rhamnolipids, with their molecular structure of hydrophilic head and lipophilic tails, most likely disrupt the outer membrane of the zoospore cell (which lacks a cell wall) due to intercalation into the membrane (Stanghellini & Miller, 1997; Kim *et al.*, 2000).

Future studies now focus on the use of surfactants in the control of *Phytophthora* diseases in tomato culture in rock wool slabs and the possible effects surfactants may have on the root tissue.

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